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Remarks

Reconsideration of this Application is respectfully requested.

The Examiner has allowed claims 27, 30-44, 50 and 52, and claim 45 is sought to be amended. Upon entry of the foregoing amendment, claims 27, 30-50 and 52 are pending in the application, with 27, 34, 39 and 45 being the independent claims. Support for the amendment to claim 45 can be found, *inter alia*, in the specification at page 10, line 2. The specification has been amended to insert replacement figures into the specification and to bring the references to the figures in the specification into conformance with the views of the replacement figures. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Objections to the Drawings

The Examiner indicated that new corrected drawings are required, citing the Notice of Draftperson's Review. (*See* Office Action at page 2, ¶ 2.) The Examiner further indicated that the corrected drawings were required in reply to the outstanding Office Action in order to avoid abandonment of the application, and that the requirement would not be held in abeyance. (*Id.*)

In view of the Notice, Applicants submit herewith replacement figures in accordance with 37 C.F.R. § 1.84. As such, Applicants respectfully request that the

Examiner acknowledge the acceptance of these drawings in any subsequent communication to Applicants.

Withdrawn Claim Rejections

Applicants thank the Examiner for the withdrawal of the objection to claim 52 and the rejection of claims 27, 30-33 and 51. (*See* Office Action at page 2, ¶¶ 3-4.)

Rejections under 35 U.S.C. § 112, First Paragraph

The Examiner rejected claims 45-49 under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement. (*See* Office Action at page 2, ¶¶ 3-4.) In particular, it is the Examiner's position that the specification,

> while being enabling for methods using type I interferons, does not reasonably provide enablement for methods using type II interferons. . . . The claims are drawn to methods of treating inflammation using interferons. However, while type I interferons share anti-inflammatory properties, the type II interferon, interferon γ, is proinflammatory. . . . Thus one of skill in the art would not predictably be able to use it as an anti-inflammatory agent.

(Office Action at pages 2-3, ¶ 5.) Applicants respectfully traverse this rejection as it may apply to the present claims.

Solely in an effort to expedite allowance of the pending claims, and not in acquiescence to the Examiner's rejection, Applicants note that claim 45 has been amended and is currently directed to a method of treating inflammatory disease in a patient comprising administering a therapeutically effective amount of a fusion protein comprising a latency associated peptide and a proteolytic cleavage site, wherein the fusion protein is covalently linked to an interferon selected from the group consisting of

interferon-α and interferon-β, and wherein the fusion protein is heterologous to the interferon.

Applicants submit that each of the members of the claimed genus of interferons has anti-inflammatory activity. Moreover, such anti-inflammatory activity has been confirmed by the art. For example, the anti-inflammatory activity of interferon-α has been confirmed in Tilg *et al.*, *Blood 85*:433-435 (1995) (Exhibit A), and the anti-inflammatory activity of interferon-β has been confirmed in Floris *et al.*, *J. of Neuroimmunology 127*:69-79 (2002) (Exhibit B).

In view of the above, Applicants submit that the claimed invention complies with the enablement requirement of 35 U.S.C. § 112, first paragraph. Accordingly, Applicants respectfully request that this rejection be withdrawn.

Allowed Claims

Applicants thank the Examiner for indicating the allowance of claims 27, 30-44, 50 and 52.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for

allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Interferon-α Induces Circulating Tumor Necrosis Factor Receptor p55 in Humans

By Herbert Tilg, Wolfgang Vogel, and Charles A. Dinarello

In the present studies we investigated the effect of interferon-α (IFNα) on the release of the soluble (extracellular) form of the tumor necrosis factor p55 receptor (TNFsRp55), because TNFsRp55 is a natural antagonist of tumor necrosis factor (TNF)-induced inflammation and also might be part of the antiinflammatory properties of IFNα. Plasma levels of TNFsRp55 were measured by a specific radioimmunoassay in five healthy volunteers and in five patients with chronic hepatitis C treated with IFNα. Levels showed a significant increase after a single injection of 5.0 million U IFNα in both healthy and hepatitis patient groups. Peak values (3.5 to 4.5 ng/mL) were observed within 12 hours of beginning treatment. Thereafter, levels promptly declined, reaching baseline values within 24 hours. TNFα and C-reactive protein (CRP) levels were below the detection limit in the same plasma samples. In addition, IFNα suppressed significantly interleukin (IL)-1α-induced TNFα protein synthesis by human peripheral blood mononuclear cells. These results suggest that the antiinflammatory properties of IFNα may be, in part, also due to the induction and/or release of TNF soluble receptors and the suppression of TNFα synthesis.

TUMOR NECROSIS FACTOR (TNF) is a pleiotropic cytokine, which possesses a wide range of biologic properties. TNF plays a major role in the pathogenesis of sepsis, the progression of acquired immune deficiency syndrome (AIDS), as well as in a number of inflammatory disorders.[1] Naturally occurring TNF inhibitors have been identified by their ability to bind to TNF and to neutralize its biologic activity.[2-4] These inhibitors were subsequently purified and sequenced, which revealed that the proteins represented the extracellular portions of the two membrane-associated TNF receptors p55 and p75. Both TNF-binding proteins are presumably derived from their respective membrane receptors by proteolytic cleavage and have, therefore, been termed soluble TNF receptors (TNFsRp55 and TNFsRp75). Soluble forms of both TNF receptors block TNF-mediated effects such as lethality in septic shock, replication of human immunodeficiency virus in vitro, and others.[4] The present study aimed to investigate whether interferon-α (IFNα) therapy is associated with the induction of circulating TNFsRp55 and whether IFNα modulates TNFα protein synthesis in vitro.

MATERIALS AND METHODS

Five healthy male volunteers and five patients with histologically and serologically confirmed chronic hepatitis C, but without evidence of cirrhosis, were included in an outpatient study with IFNα-2b (Aesca-Schering-Plough Corp, Vienna, Austria). Liver histology showed chronic persistent hepatitis in four and chronic active hepatitis in one of the patients. The protocol was approved by the Ethics Committee of the Faculty of Medicine of the University of Innsbruck and informed consent was obtained from each participant. All participants received increasing doses of IFNα subcutaneously. IFNα was injected as a single dose of 1×10^6, 3×10^6, or 5×10^6 at weekly intervals. Venous blood was drawn from each participant immediately before, and 2, 12, 24, 48, and 72 hours after the injection. Blood was collected in EDTA-containing venoject tubes placed on ice, and centrifuged within 20 minutes of venipuncture at 2,000g for 10 minutes. Plasma samples were stored at −70°C until assayed. Plasma samples obtained after injection of 1×10^6 and 5×10^6 U IFNα were assayed for TNFsRp55, TNFα and C-reactive protein (CRP).

Peripheral blood mononuclear cells (PBMC) for in vitro studies were isolated from the heparinized blood of six healthy volunteers by density centrifugation through Ficoll-Hypaque (Sigma Chemical Co, St Louis, MO). The cells were washed twice in sterile saline, and then 1 mL incubated in 5 mL polypropylene tubes at a density of 2.5×10^6 cells/mL for 24 hours in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10 mmol/L HEPES (Sigma), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Irvine Scientific, Santa Ana, CA). PBMC were incubated for 24 hours at 37°C with the same preparation of IFNα used in the patients. PBMC cultures contained 5 μg/mL polymyxin B (Pfizer Inc, New York, NY). Human recombinant interleukin (IL)-1α was kindly provided by Dr P. Lomedico (Hoffmann-LaRoche Inc, Nutley, NJ). Lipopolysaccharide (LPS) (from *Escherichia coli* 055:B5) and phorbol myristate acetate (PMA) were purchased from Sigma.

The radioimmunoassay (RIA) for the TNFsRp55 has been described.[5] This RIA is highly specific and is not affected by TNFα. The lower limit of detection is 80 to 160 pg/mL. A glycosylated form of TNFsRp55 expressed in Chinese hamster ovary cells was used to immunize New Zealand white rabbits for the generation of the anti-TNFsRp55 antibodies used in the RIA.[5] TNFα was measured by a previously described RIA.[6] The lower limit of detection of this assay is 80 pg/mL. This TNFα RIA is unaffected by the presence of TNFsRp55 at concentrations up to 10 μg/mL.[7] CRP was measured with a nephelometric method (QM 300, Kallestad, Austin, TX). The lower detection limit for CRP using this assay is 6 μg/mL. Data are presented as mean ± SE. Two-tailed paired *t*-tests were used. P values less than .05 were considered significant.

RESULTS

Pretreatment levels of TNFsRp55 in the five hepatitis C patients (1.38 ± 0.23 ng/mL) were not significantly higher from those measured in five healthy volunteers (1.12 ± 0.19 ng/mL). IFNα at a concentration of 1.0 million U did not result in an increase of circulating TNFsRp55 levels (data not shown). A higher dose (5×10^6 U), however, resulted

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in a significant induction of circulating TNFsRp55 within 12 hours in both the hepatitis C patients (3.84 ± 0.36 ng/mL, $P < .001$) and in healthy volunteers (3.76 ± 0.37 ng/mL, $P < .001$) (Fig 1). Thereafter, levels declined, reaching pretreatment values within 24 hours. TNFα and CRP levels were measured at the same time intervals as those of TNFsRp55, but were below the detection limit in each plasma sample (data not shown).

PBMC from six normal donors incubated with IFNα in concentrations of up to 1,000 U/mL or with endotoxin (100 ng/mL) did not release detectable TNFsRp55 into the supernatants after a 3-hour incubation period (data not shown). PBMC released TNFsRp55 into the supernatants after incubation with 100 ng/mL PMA for 10 minutes (0.31 ± 0.05 ng/mL, $P < .05$). PBMC incubated with increasing concentrations of IFNα (up to 1,000 U/mL) failed also to induce the synthesis of TNFα (data not shown). In contrast, IFNα suppressed IL-1-induced TNFα synthesis by 56% at a concentration of 100 U/mL IFNα ($P < .01$) and by 53% at a concentration of 1000 U/mL ($P < .01$) (Table 1).

Table 1. Suppression of IL-1α-induced TNFα Synthesis by IFNα

IL-1α (100 ng/mL)	IFNα (U/mL)	TNFα (ng/mL)
–	–	
+	–	3.2 ± 0.3
+	1	3.3 ± 0.4
+	10	2.5 ± 0.2
+	100	1.4 ± 0.2*
+	1000	1.5 ± 0.3*

PBMC were incubated for 24 hours and data show mean ± SE of PBMC from six donors. The total amount of TNFα was measured after PBMC cultures were frozen and thawed three times.
* $P < .01$.

DISCUSSION

IFNα is clinically useful in the treatment of diseases of diverse pathogenesis and manifestations. There is increasing evidence that IFNα interferes with the synthesis of various cytokines and in some cases acts as an antiinflammatory agent. IFNα also induces interleukin-1 receptor antagonist (IL-1Ra) in vitro and in vivo[8] and suppresses IL-1-induced IL-1 synthesis by PBMC.[9] The gene expression of another proinflammatory cytokine, namely IL-8, is suppressed in human fibroblasts by IFNβ.[10] Constituitive IL-8 mRNA expression in PBMC from patients with chronic myelogenous leukemia is also downregulated during IFNα therapy.[11] Several reports show that IFNα may suppress TNFα gene expression and protein synthesis.[12] Expression of the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) is mainly regulated by IL-1 and TNF. IFNα treatment of patients with chronic viral hepatitis leads to downregulation of ICAM-1 on hepatocytes.[13] In addition, IFNα has been shown to prevent endotoxin-induced mortality in mice.[14] We now show that two additional mechanisms might contribute to its anti-inflammatory properties: (1) induction of circulating TNFsRp55 during IFNα therapy and (2) suppression of IL-1α-induced TNFα synthesis in vitro by PBMC. IFN-gamma, which is not effective in the treatment of chronic viral hepatitis, does not change the amount of IL-1-induced TNFα in human PBMC[15] and is also a less potent inducer of IL-1Ra than IFNα.[8]

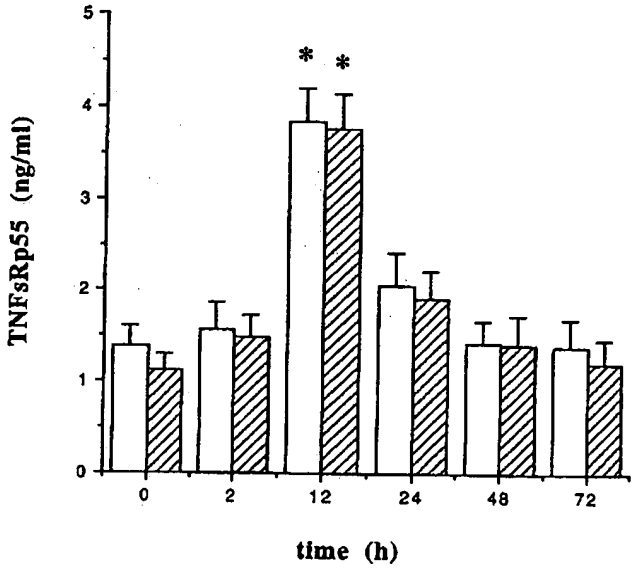

Fig 1. In vivo induction of TNFsRp55 by IFNα therapy. Patients (n = 5, □) and healthy volunteers (n = 5, ▨) received a single dose of 5.0 million U IFNα. Data are shown as mean ± SE. * indicates $P < .001$.

The induction of circulating TNFsRp55 during IFNα therapy may provide a regulatory mechanism for the modulation of endogenous circulating TNF activity. Van Zee et al recently showed that TNFsRp55 concentrations of 5 ng/mL are required to neutralize the cytotoxicity of endogenously produced TNF in the plasma of normal donors after endotoxin challenge.[16] It is difficult to establish from the published literature whether the concentrations of TNFsRp55 released into the plasma are sufficient to alter the biologic activity of the TNF circulating in various inflammatory diseases. Given the fact that the TNFsRp55 levels measured in patients receiving IFNα were approximately twofold below the peak levels achieved in endotoxin-treated normal volunteers,[5] one assumes that the TNFsRp55 circulating in IFNα-treated patients might be biologically significant. As IFNα induces both circulating IL-1 and TNF antagonists and the proinflammatory cytokines IL-1 and TNF may act as growth factors for malignant cells and several viruses, part of the clinical success of IFNα may not be only its antiinflammatory effects but also its interference with growth-promoting cytokines.

ACKNOWLEDGMENT

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Interferon-β directly influences monocyte infiltration into the central nervous system

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Abstract

Interferon-β (IFN-β) has beneficial effects on the clinical symptoms of multiple sclerosis (MS) patients, but its exact mechanism of action is yet unknown. We here suggest that IFN-β directly modulates inflammatory events at the level of cerebral endothelium. IFN-β treatment resulted in a marked reduction of perivascular infiltrates in acute experimental allergic encephalomyelitis (EAE), the rat model for MS, which was coupled to a major decrease in the expression of the adhesion molecules ICAM-1 and VCAM-1 on brain capillaries. In vitro, IFN-β reduced the mRNA levels and protein expression of adhesion molecules of brain endothelial cell cultures and diminished monocyte transendothelial migration. Monocyte adhesion and subsequent migration was found to be predominantly regulated by VCAM-1. These data indicate that IFN-β exerts direct antiinflammatory effects on brain endothelial cells thereby contributing to reduced lesion formation as observed in MS patients. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Adhesion molecules; Multiple sclerosis; Cerebral endothelium; Interferon-beta; Monocytes; Experimental allergic encephalomyelitis

1. Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS), characterized by the presence of sclerotic lesions throughout the brain. During MS, T-lymphocytes and monocyte-derived macrophages gain entry to the CNS and form perivascular infiltrates, a process which is accompanied by enhanced permeability of the blood–brain barrier (Al-Omaishi et al., 1999; Lassmann, 1997). The presence of activated monocyte-derived macrophages, filled with myelin debris, is related to disease activity and axonal damage (Brück et al., 1995, 1996). The key role for monocytes was also illustrated in the animal model for MS, experimental allergic encephalomyelitis (EAE). In this model, clinical signs and formation of perivascular infiltrates were completely abolished after the depletion of peripheral monocytes and macrophages (Huitinga et al., 1995a,b; Tran et al., 1998). These studies suggest that the infiltration of monocytes into the brain parenchyma is essential for the development of new lesions in EAE and MS.

The exact mechanism by which monocytes cross the blood–brain barrier is largely unknown. In peripheral tissues, however, the process of monocyte diapedesis has been studied in more detail and these studies demonstrated a central role for ICAM-1/CR3/LFA-1 and VCAM-1/VLA-4 interaction (Beekhuizen et al., 1993; Meerschaert and Furie, 1994). During MS and EAE, an increased expression of adhesion molecules on brain capillaries is observed (Barten and Ruddle, 1994; Cannella and Raine, 1995). Monoclonal antibodies (mAb) directed against adhesion molecules like ICAM-1, VCAM-1 and their counterligands influenced clinical signs and the formation of cellular infiltrates during EAE (Brocke et al., 1999; Engelhardt et al., 1998; Yednock et al., 1992; Baron et al., 1993; Huitinga et al., 1993; Gordon et al., 1995). Together, these studies illustrate the important role of the adhesion molecules in the development of new MS lesions.

Abbreviations: CNS, central nervous system; EC, endothelial cells; ICAM-1, intercellular adhesion molecule; MS, multiple sclerosis; VCAM-1, vascular cellular adhesion molecule; PECAM-1, platelet endothelial cellular adhesion molecule; Q-PCR, real-time quantitative polymerase chain reaction.

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So far, interferon-β (IFN-β) is one of the most successful treatments of MS proven to diminish new lesion formation as assessed by magnetic resonance imaging (Stone et al., 1997). Treatment of MS patients with IFN-β resulted also in a marked decrease in exacerbation rate and delayed sustained disease progression (Rudick et al., 1997). Previously, we showed that IFN-β treatment diminished the clinical signs of EAE animals due to a major reduction in the formation of cellular infiltrates in the CNS, whereas normal levels of encephalitogenic T-cells were found in the spleen and lymph node (Van der Meide et al., 1998; Yu et al., 1996; Ruuls et al., 1996). This suggests that IFN-β may interfere in early lesion formation by preventing the entry of leukocytes into the CNS either by influencing their migratory capacity, or by directly modulating inflammatory events at the brain capillaries.

We here demonstrate that IFN-β indeed acts directly on the brain endothelium by preventing the expression of VCAM-1 and ICAM-1, which is coupled to diminished monocyte migration as observed both in vivo and in vitro. These results also highlight that VCAM-1 is the major adhesion molecule which modulates monocyte adhesion and transmigration across brain endothelial cells during inflammation.

2. Materials and methods

2.1. Animals

For monocyte and brain endothelium isolation, male Wistar rats (250–350 g) were used. Acute EAE was induced in 8–11-week-old female Lewis rats (140–190 g). All animals were obtained from Harlan CPB (Zeist, The Netherlands) and kept under standard laboratory conditions. All experimental procedures were approved by the Experimental Animal Committee of the Vrije Universiteit.

2.2. Reagents

Ficoll–Paque gradient (Amersham Pharmacia Biotech, Sweden) and goat–α-mouse Ig-coated magnetic beads (Biomag, Polysciences, Warrington, USA) were used for monocyte isolation. The following mouse–α-rat mAb were used: OX-8 (αCD8 on T-cells), OX-19 (αCD5 on T-cells), OX-33 (αB220 on B-cells) (all IgG1 isotype), OX-52 (pan T-cell marker; IgG2a isotype) (all OX mAb, gift from Prof. Dr. D. Mason, Oxford, UK), TA-2 (αVLA-4; IgG1 isotype; Serotec, Oxford, UK), WT-1 (αLFA-1; IgG2a isotype; gift from Dr. T. Tamatani), ED7 and ED8 (αCR-3; IgG1 isotype), ED1 (monocyte marker; IgG1 isotype), ED9 (αSIRP monocyte marker; IgG1 isotype) (all ED mAb are produced in our laboratory and commercially available from Serotec), OX-6 (αMHC class II; IgG1 isotype; Serotec), 1A29 (αICAM-1; IgG1 isotype; gift from Dr. T. Tamatani), 3A12 (αPECAM-1; IgG1 isotype; donated by Prof. Dr. W.F. Hickey), 5F10 (αVCAM-1; IgG2a isotype; gift from Dr. R. Lobb, Biogen, Cambridge, USA), zona-occludens-1 (ZO-1) (goat–α-rat; Zymed Laboratories, San Francisco, CA, USA), rabbit–α-human von Willebrand Factor (vWF) (Dako, Denmark), mouse–α-smooth muscle actin clone 1A4 (Sigma, St. Louis, MO, USA) and rabbit–α-cow glial fibrillary acidic protein (GFAP) (Dako). The following conjugates were used: goat–α-mouse Ig conjugated with Alexa 594 (Molecular Probes, Eugene, OR, USA), rabbit–α-mouse $F(ab')_2$ conjugated with phycoerythrin (PE; Dako), rabbit–α-mouse IgG peroxidase (Dako) and 7-aminoactinomycin-D (7AAD) (Molecular Probes). Peroxidase activity was demonstrated by 3,3′-diaminobenzidine-tetra-hydrochloride (Sigma). Monocytes were labeled by BCECF-AM (2′,7′ bis (2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl) obtained from Molecular Probes.

Synthetic MBP 63-88 peptide was synthesized on an Applied Biosystems Peptide Synthesizer model 430 A and kindly donated by Dr. R. Weissert (Karolinska Hospital, Stockholm, Sweden) supplemented with *Mycobacterium (M) tuberculosis* type H37Ra, *M. butyricum* and incomplete Freund's adjuvant (IFA) (all from Difco Laboratories, Detroit, MI). Recombinant rat IFN-β was produced in our laboratory (Ruuls et al., 1996). Each preparation was analyzed for the absence of endotoxin contamination using *Limulus* test (Sigma). Trehalose was added for the stabilization. Recombinant rat IFN-γ was obtained from U-Cytech Utrecht, the Netherlands, and recombinant rat IL-1β was a gift of Dr. E. Kawashima, (Geneva, Switzerland).

For RNA isolation, RNA lysis buffer containing 2% β-mercaptoethanol and total isolation system were used (Promega, Madison, USA). For transcription of RNA to cDNA, reverse transcription system of Promega was used. The quantitative real-time PCR (Q-PCR) was performed using the SYBR green PCR Core reagents kit (PE Applied Biosystems, Foster City, CA, USA).

2.3. Induction and IFN-β treatment of acute EAE

Acute EAE was induced accordingly to Ruuls et al. (1996). Briefly, at day 0, rats were injected subcutaneously in both hind footpads with 0.1 ml of an emulsion containing 7.5 μl guinea pig spinal cord homogenate in PBS (1:1), 50 μg synthetic MBP 63-88 peptide, 500 μg *M. tuberculosis* type H37Ra, 50 μg *M. butyricum*, 50 μl IFA supplemented with PBS to reach a volume of 100 μl.

Animals were injected subcutaneously with IFN-β (500,000 U/animal per day) from day 6 after immunization until the end of the experiment. Control animals were injected with a substance containing similar amounts of FCS and trehalose as present in the IFN-β preparation. At the top of the disease (day 14 after immunization), the animals were sacrificed for immunohistochemical evaluation. Neurological aberrations were scored daily and graded from 1 to 4: 0, no EAE; 0.5 partial loss of tail tonus; 1, complete loss of tail tonus; 2, hind limb paresis; 3, hind limb

paralysis; 4, death. Data are expressed as means ± S.D. clinical score of seven animals per group. Data were analyzed using Student's *t*-test.

2.4. Immunohistochemistry

Untreated and IFN-β-treated rats with acute EAE were sacrificed 14 days after EAE induction. Brain and spinal cord were dissected, snap-frozen in the vapor phase of liquid nitrogen and stored at −80 °C. Cryostat sections (8 μm) were melted onto gelatin-coated glass slides and dried in containers with silica gel. Slides were fixed in acetone (10 min) and incubated with mAb against VCAM-1 (6 μg/ml) and ICAM-1 (1 μg/ml). For staining of macrophages, mAb ED1 (1 μg/ml) was used. MAb OX6 (1 μg/ml) was used to detect MHC class II expression. As secondary antibody, a rabbit–α-mouse IgG-peroxidase conjugate (1 μg/ml) was used. Peroxidase activity was demonstrated by incubation with 0.5 mg/ml 3,3′-diaminobenzidine-tetra-hydrochloride in Tris HCl buffer containing 0.03% H_2O_2. Omission of the primary mAb served as negative control.

2.5. Cerebral endothelial cell isolation

Rat cerebrovascular endothelial cells (EC) were isolated and characterized accordingly to De Vries et al. (1996). Briefly, meninges and white matter were removed from rat cortices isolated from adult male Wistar rats. The gray matter was minced and resuspended in PBS (150 mM NaCl, 10 mM $NaPO_4$, pH 7.4) containing 1 mM glucose. The suspension was incubated in 0.2% (w/v) collagenase (final concentration) in minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin for 1.5 h at 37 °C and was resuspended twice during incubation. Subsequently, the suspension was mixed with a 25% bovine serum album (BSA) (w/v) solution in PBS, up to a final concentration of 15% and centrifuged for 20 min at 800*g*. The pellet containing the cerebral capillaries was mixed with a solution of 0.1% (w/v) collagenase/dispase in MEM and incubated for 30 min at 37 °C. After incubation, capillary fragments were washed twice with cell culture medium (50% MEM, 50% F-12 nutrient mixture, supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) and seeded on culture plates coated with collagen type I. Cells were cultured until confluency was reached. Brain EC were over 98% pure as tested by von Willebrand Factor (vWF) and PECAM-1 staining. Less than 1% of the cells stained positive for pericytes (smooth muscle actin), or for astrocytic glial fibrillary acidic protein (GFAP) or microglial cells (OX42) or perivascular macrophages (ED2), as described and further characterized by De Vries et al. (1996) and Van Dam et al. (1996). Confluent brain endothelium expressed tight junctions.

The cerebrovascular EC reached confluency 5–6 days after plating and were used for studies. Confluent endothelium was pretreated with different concentrations of recombinant rat IFN-β for 24 h, followed by coincubation with or without IFNγ (200 U/ml) and interleukin-1β (IL-1β; 100 ng/ml) for different time points. Unstimulated EC served as a control.

2.6. Monocyte isolation

Rat monocytes were freshly isolated by perfusion, adapted from Scriba et al. (1996). Briefly, rats were anaesthetized by an intraperitoneal injection of 800 μl Nembutal. The thorax was opened and after an injection of 300 μl heparin (5000 IU/ml) in the heart, two intravenous canulae (BOC Ohmeda, Sweden), 16 G and 20 G, were inserted in the left ventricle (apex) and in the right ventricle, respectively. One liter prewarmed medium (RPMI-1640 supplemented with 0.5% (w/v) BSA and 20 mM HEPES (pH 7.4) was brought into the circulation and the effluent fluid was collected in tubes containing RPMI and 50 IU/ml heparin. Peripheral blood mononuclear cells (PBMC) were isolated from the concentrated cell suspension by centrifugation (400 g for 40 min at room temperature; without brake) on a Ficoll–Paque gradient ($\rho = 1.077$ g/ml). Monocytes were purified from the interface PBMC by negative selection, cells were incubated with mAb OX-8, OX-19, OX-33 and OX-52 for 30 min at 4 °C (1 μg mAb/10^6 target cells). Target cells were removed by goat–α-mouse-Ig-coated magnetic beads. Thus, 20–30 × 10^6 cells were isolated from one rat, 90% of these cells were ED9-positive monocytes. Furthermore, monocytes stained positive for VLA-4, LFA-1, CR3 and PECAM-1.

2.7. Flow cytometric analysis of adhesion molecule expression on brain endothelial cells

The brain EC were detached from the 24-well culture plates by incubation in collagenase type I (1 mg/ml). Cells were incubated with mouse–α-rat VCAM-1, ICAM-1, PECAM-1 and MHC class II mAb (1 μg/ml) for 30 min at 4 °C. Binding of these mAb was detected using PE coupled rabbit–α-mouse F(ab′)$_2$ (1 μg/ml). Omission of the primary antibodies served as negative control. Fluorescence intensity was determined using a FACScan flow cytometer (Becton & Dickinson, San Jose, CA, USA). FACS analysis was performed on viable cells, determined by 7AAD exclusion. The geometric mean fluorescence intensity of 10,000 cells was used as a measure for expression of ICAM-1, VCAM-1, PECAM-1 or MHC class II. Data are expressed as the means ± S.D. of three separate experiments. Data were analyzed using Student's *t*-test.

2.8. Real-time quantitative PCR

Cerebrovascular EC were cultured in 24-well plates. Cells were washed with PBS, lysed by adding 180 μl RNA lysis buffer containing 2% β-mercaptoethanol and stored at −70 °C. Total RNA was isolated using the SV

Total RNA Isolation system as described by the manufacturer. Concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer (Spectramax 250, Molecular Devices, USA).

RNA (0.5 μg) was reverse transcribed into cDNA using reverse transcription system with oligo-dT primers and AMV reverse transcriptase, according to manufacturer's instructions. The RT reaction was carried out at 42 °C for 1 h.

Quantitative real-time PCR (Q-PCR) was performed on cDNA in MicroAmp Optical 96-well plates with an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The reaction mixture was composed of 1x SYBR Green Buffer, 3 mM $MgCl_2$, 875 μM dNTPmix containing dUTP, 0.3 U AmpliTaq gold, 0.12 U Amperase UNG, 3.75 pmol of each primer (Table 1) and 12.5 ng cDNA with a total volume of 20 μl. The reaction conditions were as follow: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The efficiency of the primers used was tested on serial dilutions of one cDNA sample. We expect to detect two times more product of our interest gene after each cycle in the linear phase of the reaction, irrespective the concentration of cDNA used. ICAM-1 and VCAM-1 mRNA expression were quantified relatively to the level of the housekeeping GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) mRNA using the following calculation: $2^{-(\text{threshold cycle of target mRNA} - \text{threshold cycle of GAPDH mRNA})}$. The regulation factor for each treatment when compared to the control cells was calculated using the formula: average fold change (AFC)=(average of relative mRNA expression for the treatment)/(average of relative mRNA expression for the control cells). Data are expressed as the means ± S.E.M. of four wells. Data were analyzed using Student's *t*-test.

2.9. Monocyte migration

Monocyte migration was assayed using the well-established time-lapse videomicroscopy migration assay, as described previously by Van der Goes et al. (2001). Briefly, monocytes (7.5×10^5/ml) suspended in culture medium were added to brain endothelium monolayers grown in 96-well plates and allowed to migrate for 4 h. After 4 h, monolayers were placed on an inverted phase-contrast

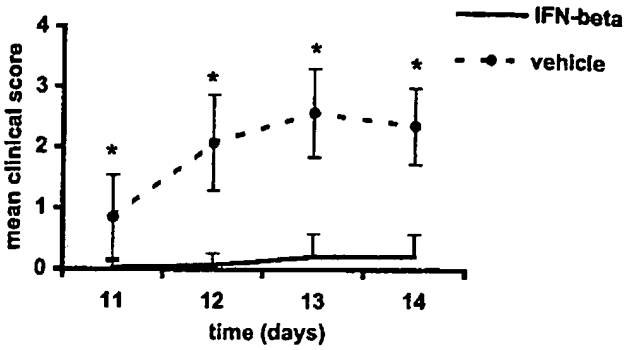

Fig. 1. IFN-β reduces clinical signs of EAE. Clinical scores (means ± S.D.) of EAE rats treated with IFN-β (500,000 U/day, subcutaneously) or vehicle ($n=7$ per group). Treatment was started at day 6 after immunization. Animals were scored daily, neurological aberrations were graded from 1 to 4; 0, no EAE; 0.5, partial loss of tail tonus; 1, complete loss of tail tonus; 2, hind limb paresis; 3, hind limb paralysis; 4, death. * Significant differences between IFN-β-treated and vehicle-treated EAE animals ($p<0.05$).

microscope (Nikon Eclipse TE300) housed in a temperature controlled (37 °C), 5% CO_2 gassed chamber (manufactured for this purpose). A field of 200 μm^2 was randomly selected and recorded for 10 min at 50 times normal speed using a color video 3CCD camera (Sony, using a CMAD2 adapter) coupled to a time-lapse videorecorder (Sony SVT S3050P). After recording, tapes were replayed at normal speed and analyzed by enumerating the number of cells within the field that had migrated through the monolayer. The migrated monocytes (phase-dark) could be readily distinguished from those remaining on top of the endothelial cell surface by their highly refractive (phase-bright) morphology. Monocyte migration was carried out either in the absence or presence of blocking mAb (10 μg/ml). Isotype matched control mAb (mouse IgG1 and mouse IgG2a) were also included. The level of migration was calculated as the percentage of migrated cells of the total monocytes within the field. Data are expressed as the means ± S.E.M. of four individual wells of three separate experiments. Data were analyzed using ANOVA followed by Student's *t*-test.

2.10. Monocyte adhesion

The adhesion assay carried out as described by Van der Goes et al. (2001). Briefly, freshly isolated monocytes (2×10^6 cells/ml) were fluorescently labeled with 0.5 μM

Table 1
Oligonucleotide primers used for the amplification of cDNAs[a]

cDNA	GenBank accession no.	Sequence (5′–3′)
Rat GAPDH	NM017008	GAACATCATCCCTGCATCCA (forward)
		CCAGTGAGCTTCCCGTTCA (reverse)
Rat ICAM-1	NM012967	GTCTGTCAAACGGGAGATGAATG (forward)
		GGTGCACGTCCCTGGTGATAC (reverse)
Rat VCAM-1	NM012889	CCTGACCTGCTCAAGTGATGG (forward)
		GAAAGAGGCTGCAGTTCCCC (reverse)

[a] Primers were designed using the Primer Express program (Applied Biosystems) and purchased from Eurogentec, Seraing, Belgium.

Table 2
The effect of IFN-β on cellular infiltration and adhesion molecule expression in spinal cord of three representative EAE rats at top of the disease

Treatment	Rat no.	Clinical score[a]	mϕ, ED1-positive	MHC class II	VCAM-1	ICAM-1
IFNβ[b]	1	1/2*	±	–	+	+
	2	0*	–	–	–	+
	4	0*	+	+	+	++
Vehicle	8	2	+++	+++	+++	+++
	10	3	++	++	++	+++
	14	3	+++	+++	+++	+++

(–) No staining. (+) 1–5 lesions positive per 2.5 mm^2. (++) 10–20 lesions positive per 2.5 mm^2. (+++) >25 lesions positive per 2.5 mm^2.
[a] On day 14, top of the disease.
[b] Days 6–14 after immunization.
* Significant difference between vehicle and IFN-β-treated EAE animals ($p<0.05$).

BCECF-AM for 15 min at 37 °C in RPMI/10% FCS and were subsequently washed with medium. Prior to the adhesion experiment, brain endothelial cell monolayers were washed twice with prewarmed RPMI medium. Subsequently, fluorescently labeled cells (1×10^6 cells/ml 1% BSA/RPMI) were added to EC monolayers and allowed to adhere for 1 h at 37 °C and 5% CO_2 in the presence or absence of blocking mAb (10 μg/ml). Isotype matched control mAb (mouse IgG1 and mouse IgG2a) were also included. After incubation, nonadherent cells were removed by gentle washing the monolayers with prewarmed RPMI/0.1% BSA. Adhered cells were lysed with 0.1 M NaOH, and fluorescence intensity was determined (Fluostar 32, BMG; excitation 485 nm, emission 535 nm). The number of adhered monocytes was calculated using a calibration curve. Data are expressed as the means ± S.E.M. of six individual wells of three separate experiments. Data were analyzed using ANOVA followed by Student's *t*-test.

3. Results

3.1. Effect of IFN-β on ICAM-1 and VCAM-1 expression in the CNS

From day 6 after EAE induction, rats were treated with IFN-β (500,000 U/day) or control vehicle. The clinical scores of IFN-β-treated EAE animals were significantly reduced compared with vehicle-treated EAE animals (Fig. 1). In the spinal cord of vehicle-treated EAE animals, perivascular cuffs with large numbers of ED1-positive macrophages were present (Table 2). In contrast, in spinal cords of IFN-β-treated EAE animals only few ED1-positive macrophage infiltrates could be detected (Table 2). Furthermore, adhesion molecule expression in the CNS of IFN-β-

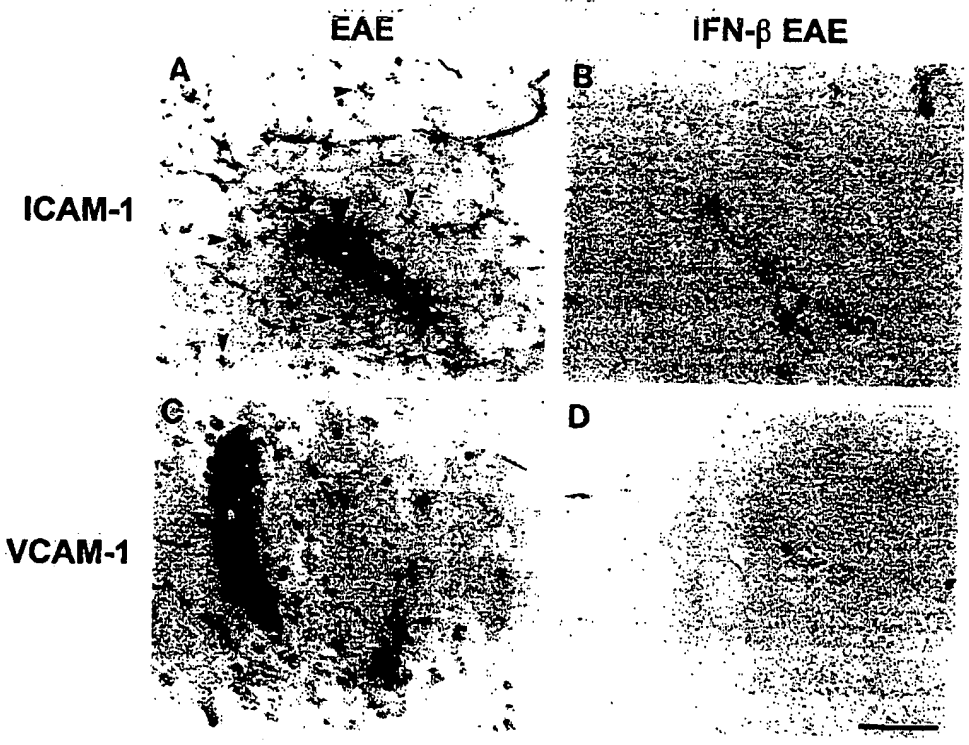

Fig. 2. Expression of ICAM-1 and VCAM-1 in the spinal cord of control and IFN-β-treated EAE animals. Representative immunohistochemical staining of spinal cord of vehicle and IFN-β-treated EAE animals. A high level of immune reactivity of ICAM-1 (A, big arrow) and VCAM-1 (C) was found on blood vessels of EAE animals. In addition, ICAM-1 was expressed by inflammatory cells and activated microglial cells (A, small arrows). A small number of ICAM-1-positive blood vessels was present in CNS of IFN-β-treated EAE animal, and the level of expression was significantly lower compared with the untreated animal (B). In the IFN-β-treated EAE animals, no detectable expression of ICAM-1 was found on microglial cells (B). VCAM-1-positive blood vessels were virtually absent in the CNS of IFN-β-treated animals (D). Bar = 16 μm (A, B), 8 μm (C) or 32 μm (D).

treated EAE animals was reduced and was similar to the expression in control brains. In the IFN-β-treated EAE animals, only a small number of ICAM-1-positive blood vessels was present and no detectable ICAM-1 expression on microglial cells was found, whereas in untreated EAE animals ICAM-1 was highly expressed by both microglial cells and brain capillaries (Table 2). A dramatic decrease of endothelial VCAM-1 expression was observed in the CNS of IFN-β-treated EAE animals, 0–5 positive lesions compared with over 20 positive lesions per 2.5 mm^2 in vehicle-treated EAE animals (Table 2). In Fig. 2, representative immunohistochemical stainings of ICAM-1 and VCAM-1 in the spinal cord of vehicle and IFN-β-treated EAE animals are shown.

3.2. Effect of IFN-β on the adhesion molecule expression on brain endothelium

To determine whether reduced expression of adhesion molecules observed in vivo is a direct effect of IFN-β, FACS analysis for ICAM-1, VCAM-1 and PECAM-1 was performed on primary brain EC. The expression of MHC class II on cerebral EC was used as a measure for cellular activation. Control primary brain EC expressed constitutively high levels of ICAM-1 and PECAM-1 and only low levels of VCAM-1 and MHC class II (Fig. 3). Treatment of the cerebral EC with proinflammatory cytokines enhanced the expression of ICAM-1 and resulted in a marked increased expression of VCAM-1 and MHC class II. Only a slightly increased expression of PECAM-1 was observed. Treatment of cerebrovascular EC with different concentrations of IFN-β for 24 h followed by coincubation with proinflammatory cytokines for 48 h prevented the upregulation of adhesion molecules in a dose-dependent manner. A maximum, but no complete, inhibition of cytokine induced expression of ICAM-1, VCAM-1, PECAM-1 and MHC class II was observed at a dosage of 1000 U/ml IFN-β (Fig. 3). Treatment of cerebral EC with 1000 U/ml IFN-β for 72 h did not influence the expression of the adhesion molecules studied. No change in cell viability was observed, as determined by 7AAD exclusion using FACS analysis.

3.3. The effect of IFN-β on ICAM-1 and VCAM-1 mRNA expression

To study whether IFN-β directly acts at the transcription level of adhesion molecules, real-time Q-PCR was performed for the mRNA encoding ICAM-1 and VCAM-1 on cerebral EC. Cytokine stimulation of brain EC resulted in a time dependent increase of mRNA levels encoding for ICAM-1 and VCAM-1. Maximal increase in mRNA encoding for ICAM-1 was observed after 2 h of cytokine

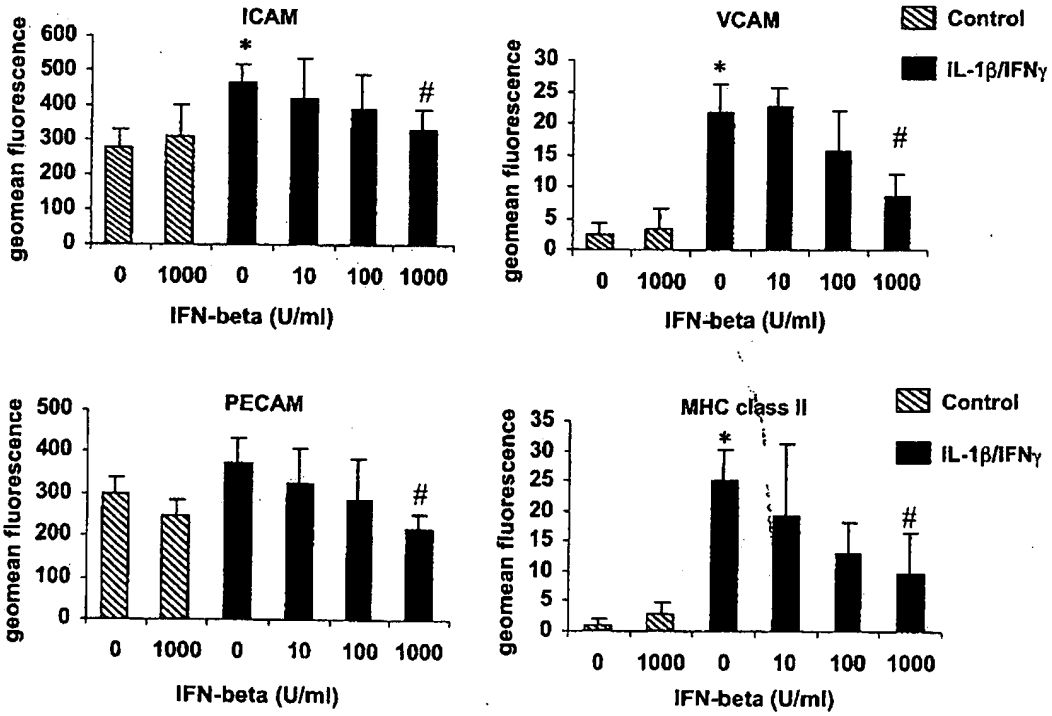

Fig. 3. IFN-β directly influences adhesion molecule expression on activated brain endothelial cells. Endothelial expression of ICAM-1 (upper left panel), VCAM-1 (upper right panel), PECAM-1 (lower left panel) and MHC class II (lower right panel) was assessed by flow cytometric analysis. Primary cerebrovascular EC were cultured for 24 h with or without IFN-β (concentrations ranging from 0 to 1000 U/ml), followed by 48-h coincubation with or without IL-1β (100 ng/ml) and IFNγ (200 U/ml). Data are expressed as the geomean fluorescence intensity and as the means of three separate experiments ± S.D. * Significant differences between control and IL-1β/IFNγ-activated endothelium ($p < 0.001$). # Significant differences between IL-1β/IFNγ-activated endothelium and IFN-β-treated cytokine-activated endothelium ($p < 0.05$).

stimulation which remained high upto 24 h and decreased after 48 h. On the other hand, mRNA encoding for VCAM-1 reached a maximum after 4 h of cytokine stimulation and decreased gradually. Treatment of cerebral EC with IFN-β for 24 h followed by cytokine costimulation resulted in a significant reduction of mRNA encoding for VCAM-1 and ICAM-1 at most time points (Fig. 4a and b). Simultaneously, reduced protein expression of ICAM-1 and VCAM-1 was observed at all time points (Fig. 4c and d). Maximum protein levels of ICAM-1 and VCAM-1 were obtained after 8 h of cytokine stimulation and these remained high upto 48 h.

3.4. Monocyte migration across brain endothelium

Since a major reduction in cellular infiltrates was observed in the CNS of EAE animals after IFN-β treatment, the effect of IFN-β on monocyte migration across cerebral endothelium was studied in vitro. Treatment of cytokine-activated brain EC with different concentrations of IFN-β reduced monocyte migration in a dose-dependent manner (Fig. 5a). In contrast, the highest dose of IFN-β did not significantly affect monocyte migration across unstimulated brain endothelium.

Since we have observed a reduced expression of VCAM-1 and ICAM-1 by IFN-β in vivo and in vitro, their subsequent role in monocyte migration was determined (Fig. 5b). In the presence of mAb directed against VCAM-1 and VLA-4, monocyte migration across activated brain endothelium was reduced by 43 ± 5.5%, whereas monocyte migration across resting cerebral EC was only slightly affected (Fig. 5b). Blocking of either VLA-4 or VCAM-1 alone reduced monocyte migration across activated endothelium to similar levels as observed when the combination of both mAb was used, 42 ± 6.4% and 49 ± 8.4%, respectively. In contrast, no significant change in monocyte migration could be detected in the presence of mAb directed against either ICAM-1, LFA-1 or CR3, or the combination of these mAb (Fig. 5b). In contrast, blocking of the PECAM-1/PECAM-1 interaction by mAb significantly

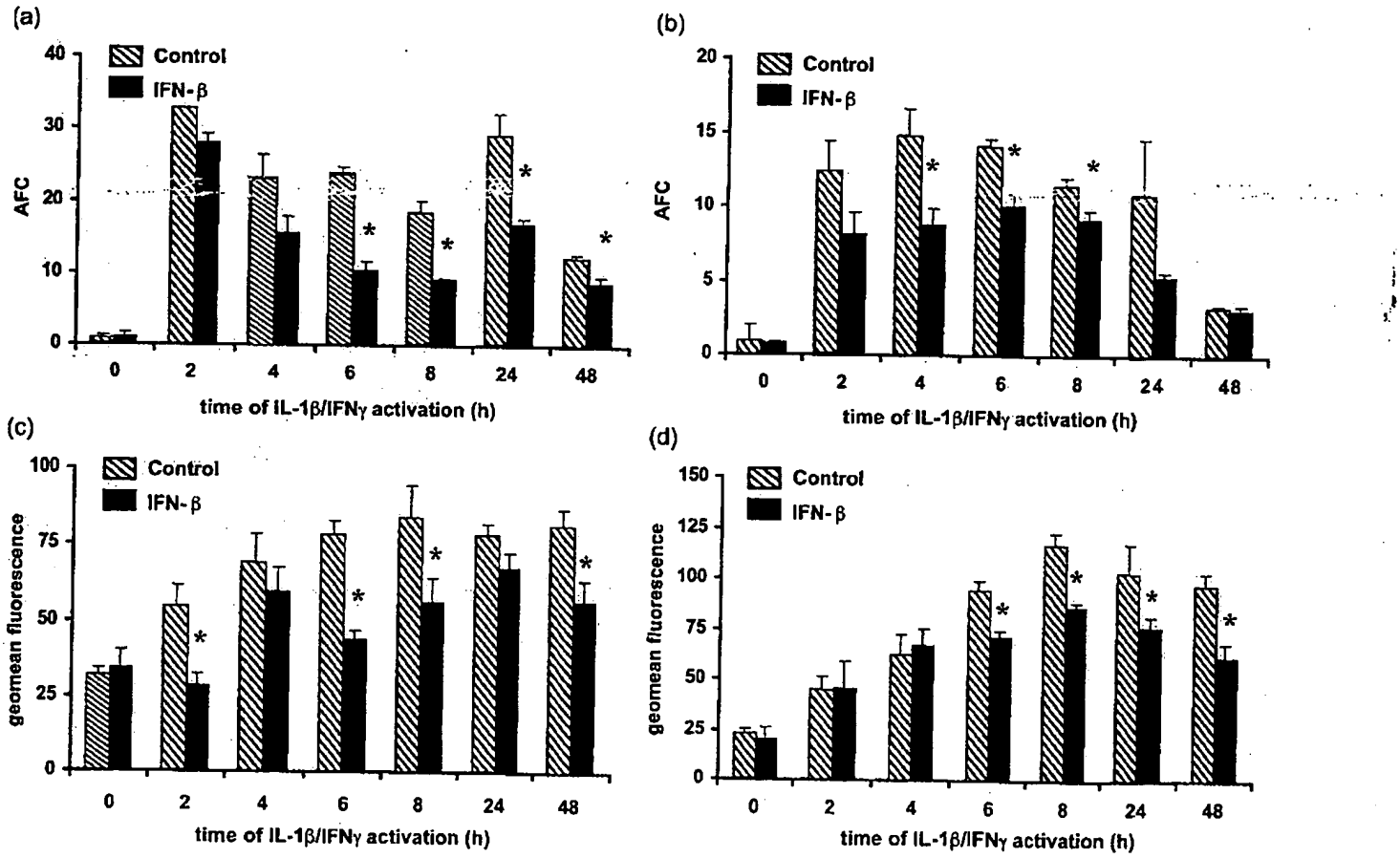

Fig. 4. IFN-β directly downregulates the mRNA levels (a, b) and expression (c, d) of ICAM-1 (a, c) and VCAM-1 (b, d) in activated brain endothelium. Primary brain endothelium was cultured for 24 h with or without IFN-β (1000 U/ml), followed by coincubation in the presence or absence of IL-1β (100 ng/ml) and IFNγ (200 U/ml) for 2, 4, 6, 8, 24 and 48 h. The ICAM-1 (a) and VCAM-1 (b) mRNA levels were quantified relatively to the level of the housekeeping GAPDH mRNA by Q-PCR. At the same time points, the protein expressions of ICAM-1 (c) and VCAM-1 (d) were analyzed by FACS analysis. The mRNA data are expressed as the means of four wells of two separate experiments ± S.E.M. RNA levels in control endothelium is set to 1, the regulation of RNA is expressed as the average fold change (AFC). The ICAM-1 and VCAM-1 expressions are expressed as the geomean fluorescence intensity and as the means of three separate experiments ± S.E.M. * Significant difference between IFN-β pretreated of IL-1β/IFNγ-activated cerebral endothelium ($p < 0.05$).

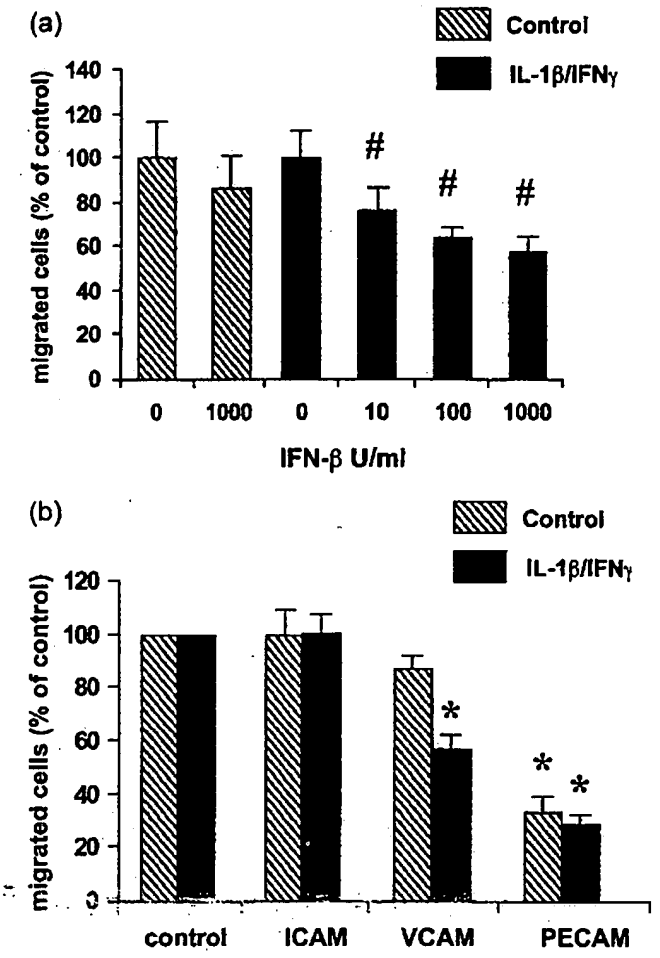

Fig. 5. Effect of IFN-β on monocyte migration across brain endothelium (a) and the role of adhesion molecules in monocyte migration (b). (a) Monocytes were allowed to migrated across cerebrovascular EC which were cultured for 24 h with or without IFN-β (concentrations ranging from 0 to 1000 U/ml), followed by 48-h coincubation with or without IL-1β (100 ng/ml) and IFNγ (200 U/ml). (b) Monocytes were allowed to migrate across monolayers of brain endothelium, which was cultured for 48 h with or without IL-1β (100 ng/ml) and IFNγ (200 U/ml). Migration was performed in absence or presence of blocking mAb: ICAM-1 indicates the presence of mAb against ICAM-1, LFA-1 and CR3; VCAM-1 indicates the presence of mAb against VCAM-1 and VLA-4; PECAM-1 indicates mAb against PECAM-1. The presence of irrelevant isotype matched control mAb did not influence monocyte migration. Results are given as a percentage of the migration across control endothelium and as the means of three separate experiments of four wells ± S.E.M. Migration across (non)stimulated endothelium served as control. For nonstimulated endothelium (hatched bars), 100% corresponds to 19 ± 1.1% migrated cells of total number of added monocytes, for stimulated EC (black bars) the 100% values corresponds with 24 ± 1.0% migrated cells of total number of added monocytes. # Significant differences between IL-1β/IFNγ-activated endothelium and IFN-β-treated cytokine-activated endothelium ($p < 0.05$). * Significant effect of blocking mAb ($p < 0.05$).

reduced the monocyte migration across both resting and activated brain endothelium by 63 ± 4.4% and 71 ± 5.3%, respectively. To exclude that the observed inhibitory effects were due to Fc receptor binding, migrations were performed in presence of isotype matched control mAb, which did not influence monocyte migration.

3.5. Monocyte adhesion to brain endothelium

Prior to transendothelial migration monocytes have to firmly adhere to brain endothelium. In order to determine whether IFN-β treatment of brain endothelium already interfered at this level, the effect of IFN-β on monocyte adhesion to cerebrovascular EC was investigated. Under control conditions, 28 ± 2.9% of the added monocytes

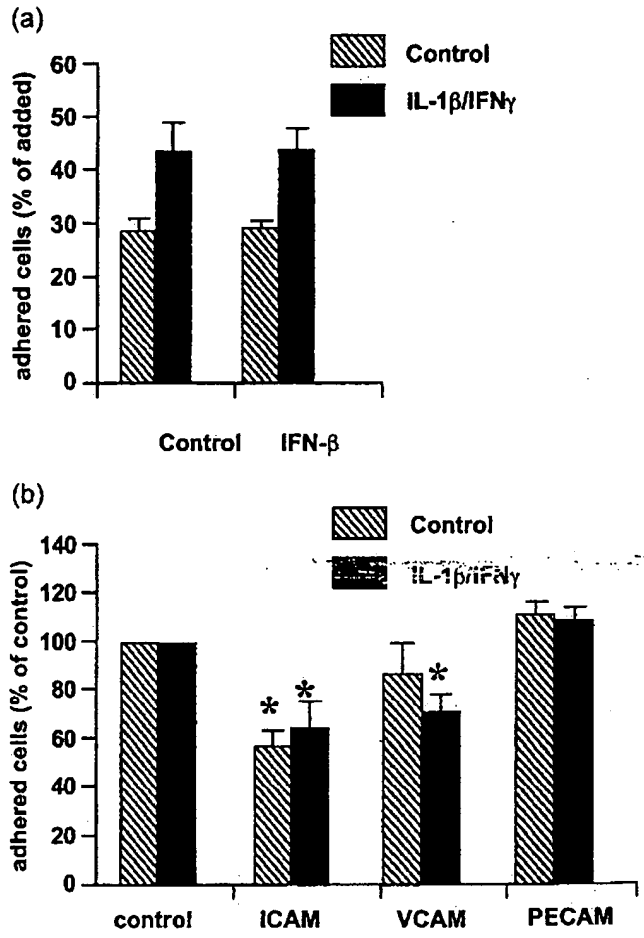

Fig. 6. Effect of IFN-β on monocyte firm adhesion to brain endothelium (a) and the role of adhesion molecules in monocyte adhesion (b). (a) Monocytes were allowed to adhere to cerebrovascular EC which were cultured for 24 h with or without IFN-β (concentrations ranging from 0 to 1000 U/ml), followed by 48 h coincubation with or without IL-1β (100 ng/ml) and IFNγ (200 U/ml). (b) Monocytes were allowed to adhere to monolayers of brain endothelium, which was cultured for 48 h with or without IL-1β (100 ng/ml) and IFNγ (200 U/ml). Adhesion was performed in absence or presence of blocking mAb: ICAM-1 indicates the presence of mAb against ICAM-1, LFA-1 and CR3; VCAM-1 indicates the presence of mAb against VCAM-1 and VLA-4; PECAM-1 indicates mAb against PECAM-1. The presence of irrelevant isotype matched control mAb did not influence monocyte adhesion. Results are given as a percentage of the added monocytes adhered to brain endothelium and as the means of three separate experiments of six wells ± S.E.M. Adhesion to (non)stimulated endothelium served as control. For nonstimulated endothelium (hatched bars), the 100% values correspond with 28 ± 2.9% adhered cells of total number of added monocytes, for stimulated EC (black bars) the 100% values correspond with 44 ± 4.3% adhered cells of total number of added monocytes. * Significant effect of blocking mAb ($p < 0.05$).

adhered to brain endothelium (Fig. 6a). After cytokine activation of brain capillaries, monocyte adhesion was increased to 44 ± 4.3%. Treatment of cerebrovascular EC with 1000 U/ml IFN-β, however, did not significantly change monocyte adhesion to control or activated endothelium (Fig. 6a).

Considering the difference in effects of IFN-β treatment on monocyte adhesion and migration, we investigated whether adhesion molecules differentially regulate monocyte firm adhesion and subsequent transendothelial migration (Fig. 6b). In the presence of mAb directed against ICAM-1, LFA-1 and CR3, monocyte adherence to control and cytokine-activated cerebral EC was significantly inhibited by 43 ± 6.7% and 33 ± 8.5%, respectively. Blocking of ICAM-1 alone reduced monocyte adhesion to control and activated brain EC by 35 ± 8.7% and 20 ± 11.9%, respectively. Monocyte adherence to activated brain endothelium in presence of VCAM-1 and VLA-4 mAb was reduced to control levels, but no significant effects of these mAb on monocyte adhesion to unstimulated endothelium could be detected. In presence of mAb directed against VCAM-1 alone similar inhibition of monocyte adhesion to activated EC (29 ± 3.2%) was observed. Blocking the PECAM-1 molecule on both brain endothelium and monocytes did not influence monocyte adhesion to control or activated EC.

Monocyte adhesion to IFN-β-treated cerebrovascular EC could still be blocked using mAb directed against ICAM-1, and mAb directed against ICAM-1 and VCAM-1 were still able to reduce monocyte adhesion to IFN-β-treated cytokine-activated endothelium (data not shown). This suggests that despite their reduced expression, these adhesion molecules were sufficiently present to mediate monocyte adhesion. The presence of irrelevant isotype matched control mAb did not influence monocyte adhesion.

4. Discussion

The present study is the first to describe that IFN-β directly exerts antiinflammatory effects on brain endothelium and influences monocyte transendothelial migration. Interestingly, monocyte firm adhesion and subsequent transendothelial migration were found to be two different processes regulated by distinct sets of adhesion molecules, illustrating a central role for VCAM-1.

At the peak of the disease in our EAE animals, an increased expression of the adhesion molecules ICAM-1 and VCAM-1 was observed on brain capillaries, and microglia were positive for ICAM-1. In contrast, in the CNS of the IFN-β-treated EAE animals, no VCAM-1 expression could be detected on the brain capillaries. In these animals, also a strong reduction of endothelial ICAM-1 expression was observed, and no ICAM-1-positive microglia were found. These findings were associated with a diminished number of cellular infiltrates of ED1-positive monocytes, as reported previously (Ruuls et al., 1996). From MRI studies, it is known that IFN-β reduces new lesion formation in MS patients (Stone et al., 1997). One of the critical events in the formation of new MS lesions is the infiltration of monocytes into the brain parenchyma. Our data suggest that IFN-β treatment may result in reduced monocyte infiltration into the CNS due to diminished adhesion molecule expression on the brain capillaries. It should be noted that besides its direct effect on brain endothelium, IFN-β may also influence the activation of leukocytes in vivo. It has been reported that pretreatment of activated leukocytes with IFN-β also inhibited transendothelial migration in a dose-dependent manner (Lou et al., 1999). Furthermore, IFN-β may reduce adhesion molecule expression on monocytes, reactive oxygen species production by monocytes and MMP9 production as described for T-cells (Lou et al., 1999; Corsini et al., 1998; Soilu-Hänninen et al., 1995; Stuve et al., 1997; Lucas et al., 1998; Calabresi et al., 2001).

To investigate whether IFN-β directly influences the inflammatory events at the level of the brain capillaries, in vitro studies were conducted. Indeed, a reduced expression of adhesion molecules was shown on primary brain EC after IFN-β treatment in vitro. Cytokine stimulation of primary cultures of cerebrovascular EC strongly induced VCAM-1, ICAM-1 and MHC class II expression in time, as reported for brain endothelial cell lines (Greenwood et al., 1995; Male et al., 1994; Pryce et al., 1997). Coincubation of cytokine-activated brain endothelium with different concentrations of IFN-β significantly inhibited the expression of ICAM-1, VCAM-1 and PECAM-1 in a dose-dependent manner. Additionally, a direct downregulation of the mRNA levels of VCAM-1 and ICAM-1 by IFN-β was assessed by Q-PCR, excluding the shedding of adhesion molecules as suggested by Kallmann et al. (2000). The observed effect of IFN-β on IFNγ induced ICAM-1, VCAM-1 and MHC class II may occur at the level of NFκB leading to reduced ICAM-1 and VCAM-1 expression (Lu et al., 1995; Van Weyenbergh et al., 1998; Pine, 1997; De Caterina et al., 2001).

Decreased adhesion molecule expression was functionally coupled to reduced monocyte diapedesis, but not to their adhesion, as no influence of IFN-β on monocyte firm adhesion was found. These findings can solely be explained by the direct influence of IFN-β on the adhesion molecule expression and their mRNA levels on the brain endothelium, as only the cerebrovascular EC were treated with IFN-β and IFN-β was absent during the experiments. So far, only minor effects of IFN-β on the adhesion molecule expression on human brain EC have been described, whereas no change in their expression on peripheral endothelium was reported (Lou et al., 1999; Corsini et al., 1998; Defazio et al., 1998; Kallmann et al., 2000; Dhib-Jalbut et al., 1996; Miller et al., 1996; Jiang et al., 1997). The observed dissimilarities in responsiveness of brain endothelium to IFN-β may be due to the different nature of peripheral endothelium and highly specialized cerebrovas-

cular EC. Alternatively, the inhibitory effect of IFN-β on endothelial adhesion molecule expression may also depend on the treatment schedule, which is different in these studies (Lou et al., 1999; Calabresi et al., 2001; Defazio et al., 1998). Other immunomodulatory therapies, like methylprednisolone, are also able to downregulate adhesion molecule expression on endothelium (Gelati et al., 2000), indicating that treatment of MS patients with these therapeutics may also act at the level of the blood–brain barrier.

The difference in effect of IFN-β on monocyte adhesion and transendothelial migration suggests that these are two differentially regulated processes. Indeed, we observed that distinct sets of adhesion molecules regulated monocyte adhesion and subsequent migration across brain EC. Transendothelial migration of monocytes across cytokine-activated brain endothelium appeared to be predominantly mediated by the VCAM-1/VLA-4 and PECAM-1/PECAM-1 pathway. On the other hand, ICAM-1 and its ligands CR3 or LFA-1 did not have a part in the transendothelial migration of monocytes. This is in contrast with reports on monocyte migration across peripheral endothelium, which is mainly regulated by ICAM-1/CR3/LFA-1 and VCAM-1/VLA-4 interaction (Beekhuizen et al., 1993; Meerschaert and Furie, 1994). In our studies, a major role was observed for PECAM-1 that appeared to mediate monocyte migration across both control and activated cerebral EC. This may be due to the fact that in our cultures PECAM-1 is mainly concentrated on the borders of the EC, thus, facilitating only the last part of the cascade of monocyte transmigration (data not shown; Muller and Randolph, 1999). Furthermore, the sets of adhesion molecules that regulate monocyte adhesion and transendothelial migration into the CNS are different from those utilized by lymphocytes. In contrast to monocyte interaction with brain EC, lymphocytes are reported to adhere to activated brain EC through LFA-1 and VLA-4, but their subsequent migration seems to be mainly regulated by ICAM-1 and partly through PECAM-1 (Baron et al., 1993; Greenwood et al., 1995; Pryce et al., 1997; Laschinger and Engelhardt, 2000; Reiss et al., 1998; Wong et al., 1999).

This study is the first to show that IFN-β exerts direct effects on the brain endothelial adhesion molecule expression, which is coupled to reduced monocyte transendothelial migration. Strikingly, distinct sets of adhesion molecules are involved in monocyte adhesion and migration across the cerebral endothelium as shown in our study, suggesting a central role for VCAM-1 in both of these processes. Therefore, it is suggested that IFN-β contributes to reduced lesion formation by modulating the inflammatory events at the blood–brain barrier, as observed in MS.

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